EFFECT OF RIPENING TIME AND THE COMBINATION OF EWE AND GOAT MILK ON THE MICROFLORA OF PICANTE CHEESE

EFFETTO DEL TEMPO DI MATURAZIONE E DELLA MISCELAZIONE DI LATTE DI PECORA E DI CAPRA SULLA MICROFLORA DEL FORMAGGIO "PICANTE"

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ABSTRACT

Changes in the counts of total viable microorganisms, lactic acid bacteria, Enterobacteriaceae, staphylococci, and yeasts, as well as pH, salt content, total solids content, ash content, and a_w of Picante were examined using ANOVA. Five equally spaced proportions of goat to ewe milk covering the range of 0 to 100% were tested, and the corresponding cheeses were sampled at increasingly spaced times over a 180-day ripening period. Numbers of staphylococci and Enterobacteriaceae were very high, especially in the first days and for cheeses made with a high-

RIASSUNTO

Nel presente lavoro sono stati esaminati i cambiamenti osservati nel formaggio Picante in merito alle conte di microrganismi vitali, batteri lattici, Enterobacteriaceae, stafilococchi e lieviti. Sono stati inoltre indagati il valore di pH, il contenuto di sali, di solidi totali, di ceneri e a_w usando ANOVA. Sono stati saggiati campioni di latte di capra aggiunto in cinque diverse proporzioni, da 0 a 100%, al latte di pecora. Partendo da queste differenti miscele di latte, i rispettivi formaggi sono stati campionati a tempi di maturazione crescenti, fino a 180 giorni. Il nume-

- Key words: microbial flora, mixture of ewe and goat milk, physico-chemical factors, Picante cheese, ripening time. -

er percentage of ewe milk. The major groups present throughout ripening were lactic acid bacteria and yeasts. Strains of lactobacilli were ubiquitously present, and their disappearance between 130-180 days seems to be related to the increase in NaCl content up to 12% (w/w). There was a notable increase in the levels of yeasts during the first days, and a significant decrease towards 130 days. Both milk composition and ripening time had statistically significant effects in all microbiological and physico-chemical variables measured, but milk composition apparently did not cause significant differences in terms of overall organoleptic evaluation. Attempts to mathematically correlate the numbers of microorganisms with time were successfully performed.

ro di Stafilococchi e di Enterobacteriaceae è risultato molto alto, in particolare nei primi giorni e per formaggi con una alta percentuale di latte di pecora. I gruppi maggiormente presenti durante la maturazione sono stati i batteri lattici ed i lieviti. Colonie di lattobacilli sono state ubiquitariamente riscontrate e la loro scomparsa tra i 130 e i 180 giorni sembra essere collegata all'incremento di NaCl che arriva fino al 12% in peso. È stato notato un notevole incremento del livello dei lieviti durante i primi giorni e un significativo calo verso i 130 giorni. Sia la composizione del latte che il tempo di maturazione hanno evidenziato effetti statisticamente significativi su tutti i microrganismi e sulle variabili chimico-fisiche misurate; tuttavia la composizione del latte non ha causato, apparentemente, cambiamenti significativi in termini di valutazione organolettica globale. Sono stati infine effettuati, con risultati positivi, dei tentativi di correlazione matematica tra il numero dei microrganismi e il tempo di maturazione.

INTRODUCTION

Although artisanal cheeses made from milk of small ruminants such as sheep and goats account for a very small proportion of the worldwide cheese production, such cheeses have important social and economic impact in Mediterranean countries such as Portugal, Spain, France, Italy and Greece. The organoleptic and textural uniqueness of the cheeses manufactured with ewe and goat milk have attracted the attention of several researchers in recent years (CARBALLO et al., 1994; DEIANA et al., 1977; FATICHENTI et al., 1979; FERNANDEZ DEL POZO et al., 1988a,b; FONTECHA et al., 1990; GOMEZ et al., 1989; LLANO et al., 1992; NUÑEZ et al. 1978). They attempted to correlate the specific properties observed in the cheeses with the microstructure of the casein micelles and fat globules in the milk, on the one hand, and with the native (or contamination) microflora, on the other. Such attempts are driven by the goal to standardize the manufacture of these cheeses, a *sine qua non* condition for the survival of such cheeses in the increasingly demanding world market.

Picante da Beira Baixa (or simply Picante) cheese, a hard, salty, and spicy cheese originating in Portugal, is manufactured manually in small quantities

from a mixture of raw ewe and goat milk using animal rennet without deliberate addition of any starter culture (CRUZ et al., 1945).

Ripening of Picante differs from most cheeses in that the curd is rubbed with dry salt at the time of making it and the cheeses are maintained in the maturation room for 2 to 8 days, rubbed once again with salt, grouped in small horizontal sets of 2 or 3 cheeses, and left in this manner for 2 to 3 weeks.

They are eventually brought together in vertical sets of several cheeses supported by intercalated layers of clean sand and wheat straw, and maintained in that way for a minimum of 4 months with periodic washing with tap or salt water.

The objective of the present study was to quantitatively characterize the development of the major microbial families (lactic acid bacteria, yeasts, Enterobacteriaceae, and staphylococci) and several physico-chemical properties (pH, NaCl content, total solids content, ash content, and a_w) which play a role in microbial development throughout the ripening period.

Establishment of relationships between physico-chemical properties and microbial development was attempted using semiempirical models, and the effect of different proportions of ewe and goat milk on such development was ascertained using ANOVA. This research effort is important because virtually no data are available to date on Picante cheese, although the issue of the microbial safety of this cheese has been raised in the mass media several times in the recent past. On the other hand, it is likely that extensive insight into the microecology of Picante cheese (supported by suitable methods of assessment of statistical significance thereof) will lead to rational improvements of the farmhouse methods for manufacturing this unique cheese.

MATERIALS AND METHODS

Manufacture, experimental design, and sampling

Five batches of mixtures of Charnequeira goat and Merino ewe milk with the compositions 0/100 (goat/ewe), 25/75, 50/50, 75/25, and 100/0 %(v/v), respectively, were prepared. These batches will be denoted hereafter as 0C, 25C, 50C, 75C and 100C, respectively.

Each batch was used to manufacture 20 cheeses according to the traditional procedure, and they were all ripened *in situ* under similar environmental conditions.

Ten cheeses (two cheeses per batch) randomly taken were sent under refrigerated conditions to our laboratory at 0, 9, 25, 40, 55, 83, 110, 140, and 180 d of ripening (counted from the time of the salting procedure), and radial slices were randomly taken from each cheese and used for analysis (hence, a replicated 5x9 factorial design was used). A replicated 5x6 factorial design was used for Enterobacteriaceae counts and a replicated 5x8 factorial design was used for yeast counts. For the pH analysis, a replicated 5x9 factorial design was used for both the outer pH (upper portion of slices) and the inner pH (interior portion of slices).

Microbiological analyses

The cheese samples (10g) were homogenized with 90 mL of sterile 2% (w/v) sodium citrate (Merck, Frankfurt, Germany) at 45°C for 3 min in a stomacher Lab-Blender 400 (Seward Medical, London, U.K.).

Sequential decimal dilutions of the cheese homogenate were made with sterile 0.1% (w/v) peptone water (Sigma, St. Louis, MO, U.S.A) and plated in duplicate on a range of media: total viable counts on Plate Count Agar (PCA) (Lab M, Bury,

U.K.) incubated at 30°C for 3 d under aerobic conditions; lactococci on M17A Agar (M17A) (Lab M) incubated at 30°C for 3d; lactobacilli on Rogosa Agar (RA) (Oxoid, Basingstoke, U.K.) incubated at 30°C for 5d; yeasts and moulds on Potato Dextrose Agar (PDA) (Lab M) incubated at 25°C for 5d; coagulase positive staphylococci (*Staphylococcus aureus*) on Baird Parker Medium (BPM) (Lab M) supplemented with egg yolk tellurite (Lab M), incubated at 37°C for 2d; and Enterobacteriaceae on Violet Red Bile Glucose Agar (VRBGA) (Lab M) incubated at 37°C for 1d.

The M17A and RA were supplemented with cycloheximide (100 mg/L) (Sigma) to prevent yeast growth, and sterile 10% (v/v) lactic acid (Merck) was added to the PDA to improve its selectivity.

All microbiological counts were made according to the surface viable count technique of MILES and MISRA (1938), except for VRBGA counts which were determined by the pour-plate technique of BUSTA et al. (1984). The results are expressed as cfu/g of cheese.

Physico-chemical analyses

The analyses were done according to RICHARDSON (1985) and KOSIKOWSKI (1982). The total solids content was determined by the oven method (100°C) and the pH values were measured with an electrode for solids (Ingold, Urdorf, Switzerland) connected to a potentiometer Crison Microph 2001 (Crison, Barcelona, Spain).

The NaCl content was determined by the modified Volhard method using silver nitrate and potassium thiocyanate (Merck); the water activity (a_w) was measured with a model DP989M Protimeter dewpoint meter (Protimeter plc, Bucks, U.K.), and the ash content was determined according to the AOAC method (1990).

Sensory analyses

Cheeses ripened for 180 d were assessed organoleptically by a group of 9 experienced panelists. Cheeses were graded for form (0 to 4; 0 for very bad and 4 for very good), rind (0-4), texture (0-6) and flavour (0-6), and the scores were summed to give a quantitative value for the overall sensorial appreciation.

RESULTS

Experimental data

The experimental data pertaining to the microbial counts for various times during the ripening period and various proportions of ewe and goat milk are plotted three-dimensionally in Figs. 1-6. The experimental data pertaining to the pH values, salt content, total solids content, ash content, and water activity are reported in Table 1. The numerical data pertaining to the overall sensory appreciation are reported in Table 2.

Statistical analyses

For each microbiological and physico-chemical variable, two-way analyses of variance (ANOVA) were carried out. The use of ANOVA is valid if the experimental errors are independent and normally distributed, and if they possess a constant variance; for all variables except for pH and NaCl content the original experimental data had to be transformed using a λ-power form so as to achieve normality (BOX et al., 1978). The more important information conveyed by the ANOVA tables containing the (un)transformed data is summarized in Tables 3 and 4. Plots of the residuals of the transformed data (not shown) indicate that the behavior of the errors of the transformed data is in good agreement with the assumptions underlying the validity of the

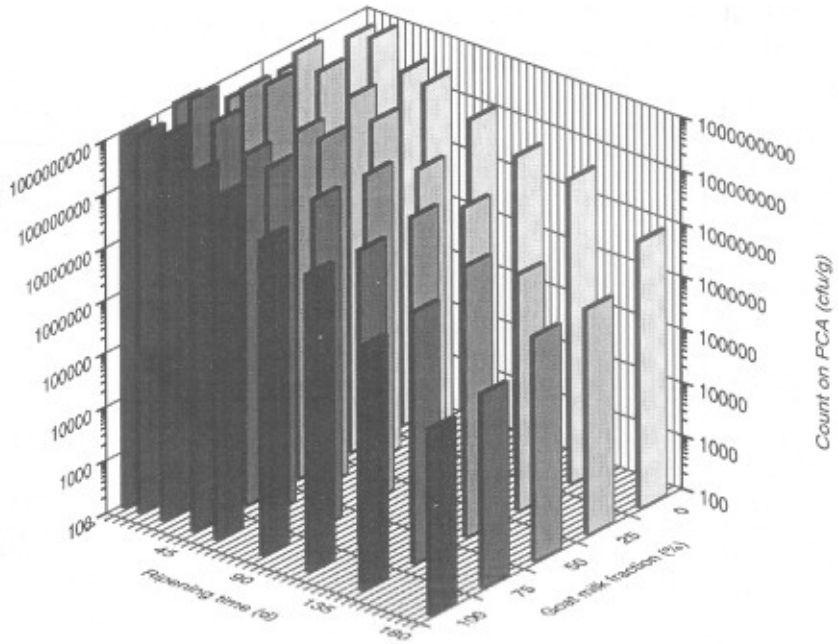

Fig. 1 - Three-dimensional plot of microbial counts on PCA vs. ripening time and volumetric fraction of goat milk.

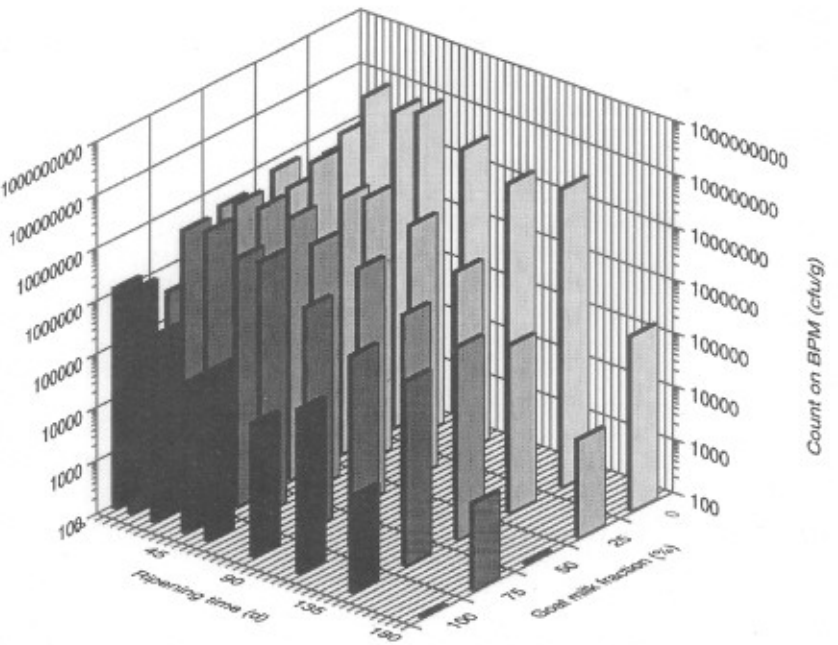

Fig. 2 - Three-dimensional plot of microbial counts on BPM vs. ripening time and volumetric fraction of goat milk.

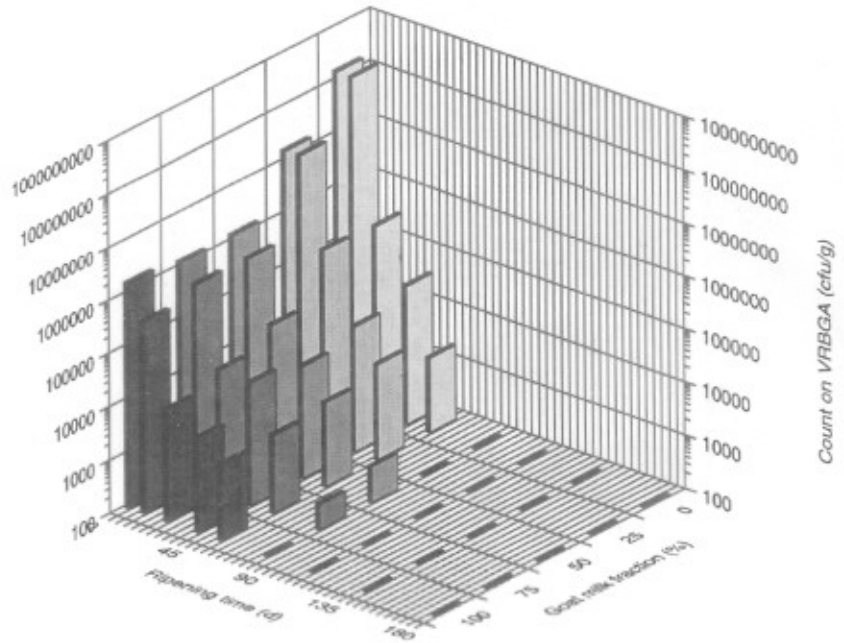

Fig. 3 - Three-dimensional plot of microbial counts on VRBGA vs. ripening time and volumetric fraction of goat milk.

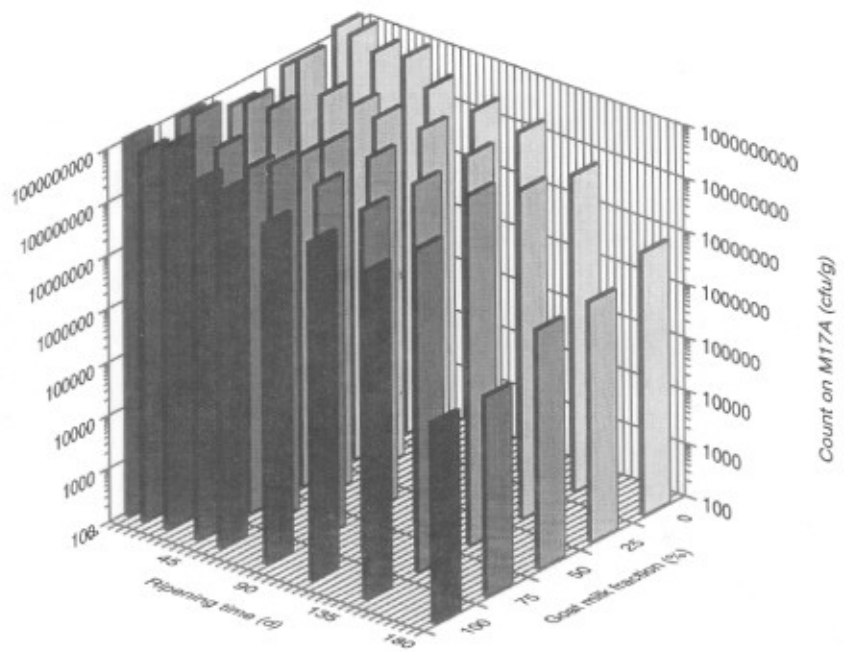

Fig. 4 - Three-dimensional plot of microbial counts on M17A vs. ripening time and volumetric fraction of goat milk.

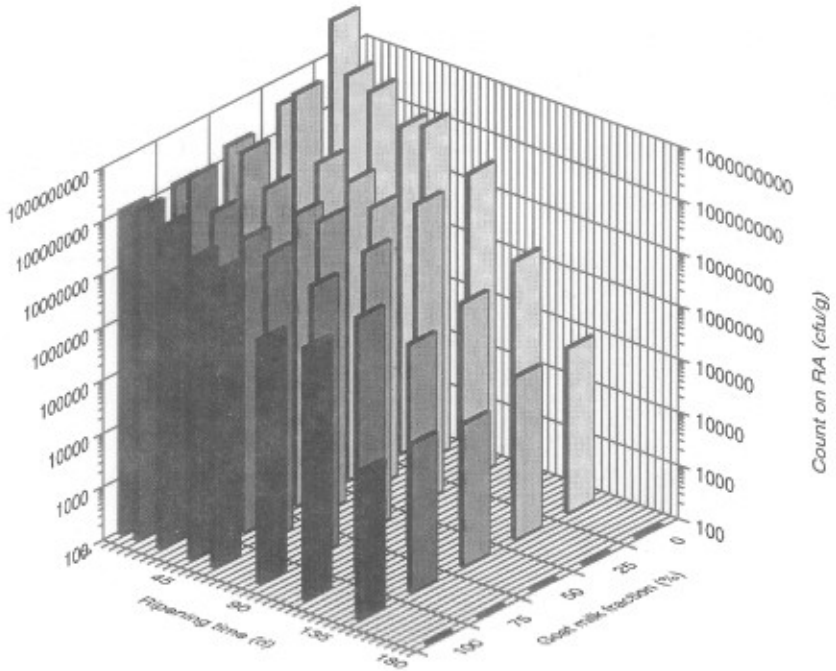

Fig. 5 - Three-dimensional plot of microbial counts on RA vs. ripening time and volumetric fraction of goat milk.

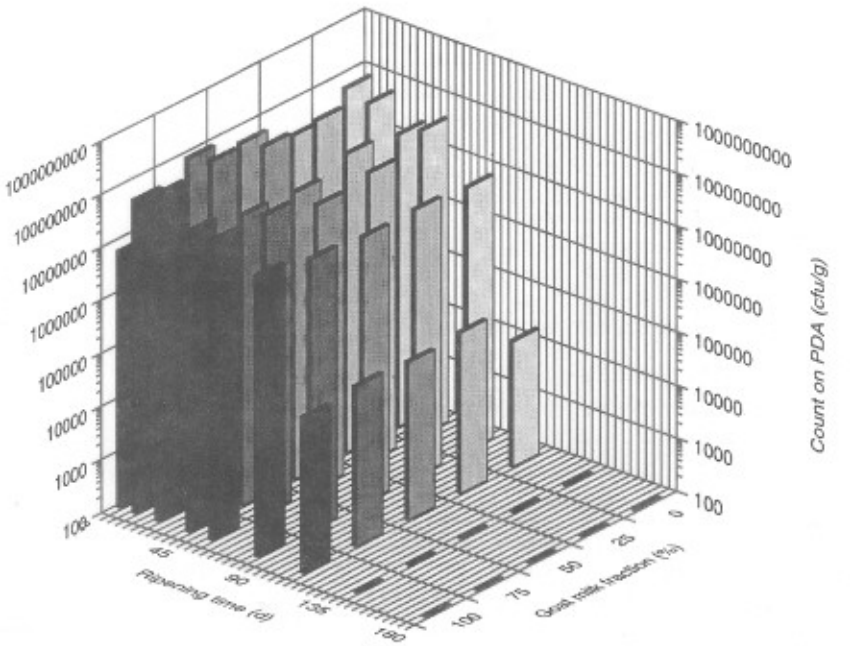

Fig. 6 - Three-dimensional plot of microbial counts on PDA vs. ripening time and volumetric fraction of goat milk.

Table 1 - Mean values, throughout ripening, of physico-chemical variables for the five batches of Picante cheese with different goat and ewe milk compositions.

Physico-chemical property	Type[1] of cheese	Ripening time (d)								
		0	9	25	40	55	83	110	140	180
inner pH	0C	5.37	4.97	5.27	5.18	5.19	5.33	5.66	5.81	5.77
	25C	5.37	5.18	5.46	5.24	5.12	5.34	5.73	5.95	5.89
	50C	5.45	4.56	5.11	5.04	5.03	5.35	5.63	5.85	5.83
	75C	5.05	4.49	5.05	5.06	5.09	5.28	5.75	5.96	5.80
	100C	4.95	4.55	4.82	5.01	5.04	5.33	5.70	5.86	5.79
outer pH	0C	5.93	5.64	5.70	5.80	5.66	6.10	6.63	6.29	6.14
	25C	5.79	5.79	5.74	5.78	5.62	6.06	6.94	6.22	6.29
	50C	5.70	4.95	5.54	5.55	5.52	6.17	6.53	6.23	6.27
	75C	5.51	4.74	5.53	5.54	5.41	6.15	6.53	6.22	6.32
	100C	5.39	4.95	5.33	5.60	5.44	6.06	6.39	6.16	6.28
NaCl (%)	0C	3.08	8.06	7.48	7.73	8.14	8.07	8.60	8.72	11.83
	25C	3.96	7.60	8.02	7.94	7.82	8.07	8.78	8.69	11.59
	50C	3.74	7.01	7.71	7.80	8.28	8.48	8.85	9.15	11.82
	75C	3.55	8.13	6.96	7.86	7.74	8.13	8.43	8.42	11.50
	100C	3.00	8.36	7.74	7.53	7.74	8.48	8.29	8.33	11.76
a_w	0C	0.96	0.92	0.94	0.92	0.92	0.92	0.91	0.87	0.78
	25C	0.98	0.93	0.92	0.92	0.92	0.92	0.90	0.87	0.78
	50C	1.00	0.95	0.95	0.93	0.92	0.91	0.92	0.87	0.78
	75C	0.97	0.93	0.95	0.92	0.93	0.91	0.91	0.85	0.77
	100C	0.96	0.91	0.93	0.93	0.92	0.91	0.90	0.87	0.78
total solids (%)	0C	40.33	46.33	49.83	51.06	51.50	50.96	52.41	56.70	59.52
	25C	40.10	48.63	50.07	49.79	50.16	51.46	52.59	56.16	59.51
	50C	42.26	47.75	49.71	48.77	50.08	50.04	52.32	55.19	59.72
	75C	42.73	49.25	49.72	52.19	51.54	50.38	52.58	56.31	61.16
	100C	44.01	50.63	50.80	50.05	51.77	50.64	53.44	57.58	59.97
ash (%)	0C	5.15	7.90			8.40				12.75
	25C	4.60	7.75			8.75				13.00
	50C	4.95	7.30				9.65			12.65
	75C	4.25	10.05				12.85			9.15
	100C	3.70	9.80				9.05			12.50

[1] The type of cheese refers to the volumetric percentage of goat milk.

ANOVA methodology, and so the statistical analysis can be validated.

For the organoleptic data, Student's *t*-tests of the hypothesis that the average total score for the cheeses is different for different milk compositions were implemented using appropriate estimates of variance pooled from every pair of data sets under consideration. The null hypothesis, i.e. that the overall organoleptic score of the cheeses is essentially the same irrespective of milk composition, was accepted in all cases at the 5% level of significance.

Table 2- Scores for the overall organoleptic appreciation of the five bacthes of Picante cheese with different goat and ewe milk compositions. The overall scale is 0 - 20, and is obtained as the sum of the scales used to score the cheese form (0-4), rind (0-4), texture (0-6), and flavour (0-6), where the lower and upper scores associated with each sensorial attribute denote very bad and very good, respectively.

Panelist Number	Type of cheese[1]				
	0C	25C	50C	75C	100C
1	16.5	17.0	15.0	14.0	16.5
2	18.0	15.5	17.0	19.0	17.0
3	15.0	18.0	14.5	16.5	17.0
4	14.5	17.0	14.5	17.5	15.5
5	14.0	13.0	12.5	14.5	15.0
6	16.5	16.5	15.5	17.5	17.5
7	14.0	17.0	18.0	18.0	16.0
8	14.0	16.0	15.0	16.0	13.0
9	12.0	13.0	11.5	15.0	16.0

[1] The type of cheese refers to the volumetric percentage of goat milk.

Table 3 - ANOVA table for the replicated factorial arrangement pertaining to the microflora enumerated on the various media after transformation of the data.

Enumerating medium	λ	Source of variation	Ratio of mean squares*
PCA	-0.04	Factor C	16.337
		Factor T	702.97
		Interaction CT	13.267
BPM	-0.06	Factor C	1,721.1
		Factor T	2,567.3
		Interaction CT	243.56
VRBGA	0.16	Factor C	356.62
		Factor T	1,499.3
		Interaction CT	99.854
M17A	-0.0025	Factor C	5.0750
		Factor T	409.41
		Interaction CT	6.9960
RA	-0.1	Factor C	45.139
		Factor T	19,791
		Interaction CT	8.7500
PDA	-0.1	Factor C	26.691
		Factor T	15,036
		Interaction CT	12.698

λ: Maximum likelihood estimator.
C: Composition; T: Time; CT: Composition and Time.
·All values were significant at the 0.1% level of significance.

Table 4 - ANOVA table for the replicated factorial arrangement pertaining to physico-chemical parameters.

Physico-chemical parameters	Source of variation	Ratio of mean squares
Inner pH	Factor C	163.26*
	Factor T	1,3807*
	Interaction CT	33.523*
Outer pH	Factor C	65.357*
	Factor T	427.86*
	Interaction CT	11.643*
NaCl (%)	Factor C	2.6774
	Factor T	712.90*
	Interaction CT	3.3952*
total solids (%)	Factor C	14.303*
	Factor T	631.84*
	Interaction CT	3.7060*

C: Composition; T: Time; CT: Composition and Time
* Significant at the 0.1% level of significance.

Mathematical analyses

In the case of the correlations of the populations of the different microbial families with observable physico-chemical parameters, the following simple model was used:

$$-\frac{dC_X}{dt} = (\mu_{o,d} + (\mu_{c,d} - \mu_{o,d})\, y_c)\, C_X \qquad (2)$$

where C_X is the concentration of viable microflora (in cfu/g), t is the time elapsed after the salting procedure, $\mu_{o,d}$ and $\mu_{c,d}$ the specific death rates in cheeses made with 100% ewe milk and 100% goat milk, respectively, and y_c the volumetric percent of goat milk. It is commonly accepted (SPERBER, 1983) that the specific rate of death increases when the water activity of the medium decreases; in its simpler form, they should be related by

$$\mu_{o,d} = \alpha_{o,0} - \alpha_{o,1}\, a_{o,w}$$
$$\mu_{c,d} = \alpha_{c,0} - \alpha_{c,1}\, a_{c,w} \qquad (3)$$

where $\alpha_{o,0}$, $\alpha_{o,1}$, $\alpha_{c,0}$, and $\alpha_{c,1}$ are positive parameters, and $a_{o,w}$ and $a_{c,w}$ are the water activities in cheeses made from 100% ewe milk or 100% goat milk, respectively. In addition, it has been found (FERNÁNDEZ SALGUERO and LLINARES, 1985; GÓMEZ and FERNÁNDEZ SALGUERO, 1992; MARCOS et al., 1981; ROBINSON and STOKES, 1959) that the water activity correlates linearly with the molality of salt according to

$$a_{o,w} = \beta_{o,0} - \beta_{o,1}\, m_{o,s}$$
$$a_{c,w} = \beta_{c,0} - \beta_{c,1}\, m_{c,s} \qquad (4)$$

where $\beta_{o,0}$, $\beta_{o,1}$, $\beta_{c,0}$, and $\beta_{c,1}$ are positive parameters, and $m_{o,s}$ and $m_{c,s}$ denote the molality of salt in cheeses made from 100% ewe milk and 100% goat milk, respectively. Finally, fundamental considerations pertaining to the movement of salt by diffusion from the surface of the cheeses (essentially kept under saturated conditions of salt at all times) into the bulk (CRANK, 1975) within the time frame utilized in our experiments and under the approximate assumption that the cheese behaves as a semi-infinite medium have led the following approximate relationship to be proposed for the molality of salt, $\bar{m}_s$, averaged over the entire cheese and over time t:

$$\bar{m}_{o,s} = \gamma_{o,0} + \gamma_{o,1}\sqrt{t}$$
$$\bar{m}_{c,s} = \gamma_{c,0} + \gamma_{c,1}\sqrt{t} \qquad (5)$$

where $\gamma_{o,0}$, $\gamma_{o,1}$, $\gamma_{c,0}$, and $\gamma_{c,1}$ are positive parameters, and $\bar{m}_{o,s}$ and $\bar{m}_{c,s}$ are the molality of salt averaged over the entire cheese and time t for cheeses made from 100% ewe milk and 100% goat milk, respectively. (In this equation, the γ_0's are proportional to the initial salt content of the cheese, whereas the γ_1's are proportional to the product of the saturation concentration at the surface of the cheese by the square root of the apparent diffusivity of salt within the cheese matrix). Combining eqs. (2)-(5) yields, upon integration, the following relationship:

$$\ln\left\{\frac{C_X}{C_{X,0}}\right\} = -(\varepsilon_0 + \varepsilon_1\, y_c)\, t - (\varepsilon_2 + \varepsilon_3\, y_c)\, t^{3/2} \qquad (6)$$

where parameters ε_0, ε_1, ε_2, and ε_3 are defined as $\varepsilon_0=\alpha_{o,0}-\alpha_{o,1}(\beta_{o,0}-\beta_{o,1}\gamma_{o,0})$, $\varepsilon_1=\alpha_{o,0}-\alpha_{c,0}+\alpha_{c,1}\beta_{o,0}(1-\gamma_{o,0})+\alpha_{o,1}(\beta_{c,1}\gamma_{c,0}-\alpha_{o,1}\beta_{c,0})$, $\varepsilon_2=2\alpha_{o,1}\beta_{o,1}\gamma_{o,0}/3$, and $\varepsilon_3=2(\alpha_{o,1}\beta_{c,1}\gamma_{c,1}-\alpha_{c,1}\beta_{o,1}\gamma_{o,1})/3$, respectively.

The experimental data overlaid on the corresponding theoretical model of the form given by eq.(6) are depicted in Figs. 7-9 for some microbial groups. The best estimates for the ε parameters (calculated by nonlinear regression analyses) are tabulated in Table 4. Plots of all residuals of the experimental data with respect to the values predicted by the model given by eq.(6) (not shown) do not display biased trends, whereas plots of the same

residuals against the percentiles of a normal distribution do not depart significantly from linearity; hence, no apparent statistical reason exists to doubt the form of the models proposed, and consideration of other functional forms and/or inclusion of pH-dependencies was not pursued.

DISCUSSION

High numbers of total viable microorganisms (PCA), i.e. above 10^8 cfu/g, were detected throughout the ripening period for all cheeses, showing an increase during the first days of ripening followed by considerable decreases until 180 d of

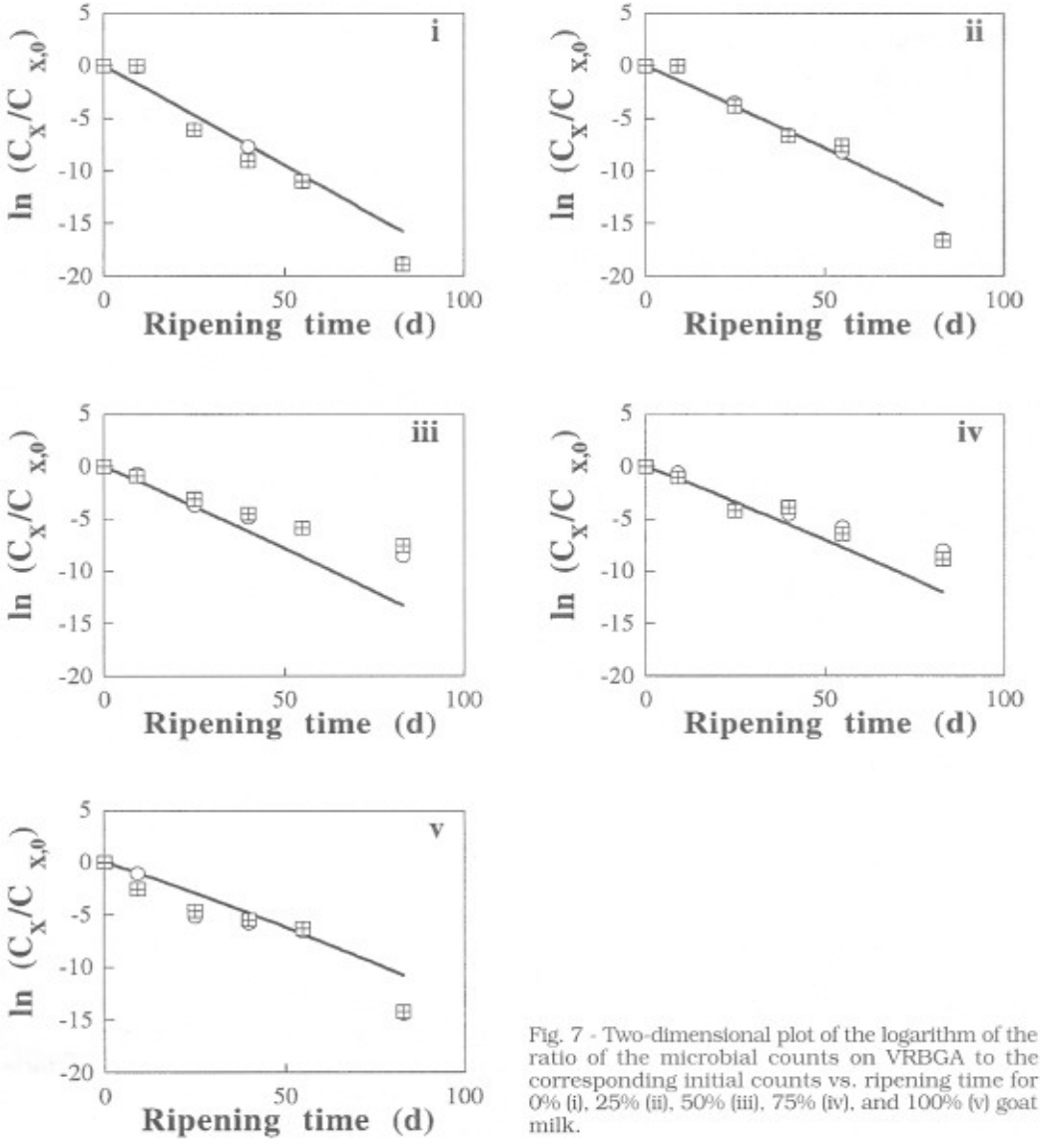

Fig. 7 - Two-dimensional plot of the logarithm of the ratio of the microbial counts on VRBGA to the corresponding initial counts vs. ripening time for 0% (i), 25% (ii), 50% (iii), 75% (iv), and 100% (v) goat milk.

ripening (see Fig. 1). These results are similar to those previously reported by other authors who have worked with artisanal cheeses made from goat and ewe milk (FONTECHA et al., 1990; GAYA et al., 1983; LLANO et al., 1992; NUÑEZ et al., 1978).

The numbers of staphylococci (BPM) and Enterobacteriaceae (VRBGA) were relatively high (10^6 and 10^8 cfu/g, respectively), especially during the first days of ripening and for the 0C and 25C cheeses (Figs. 2 and 3). This observation is an indication of the poor microbiological quality of the raw milk, particularly the ewe milk. In all cheeses, the

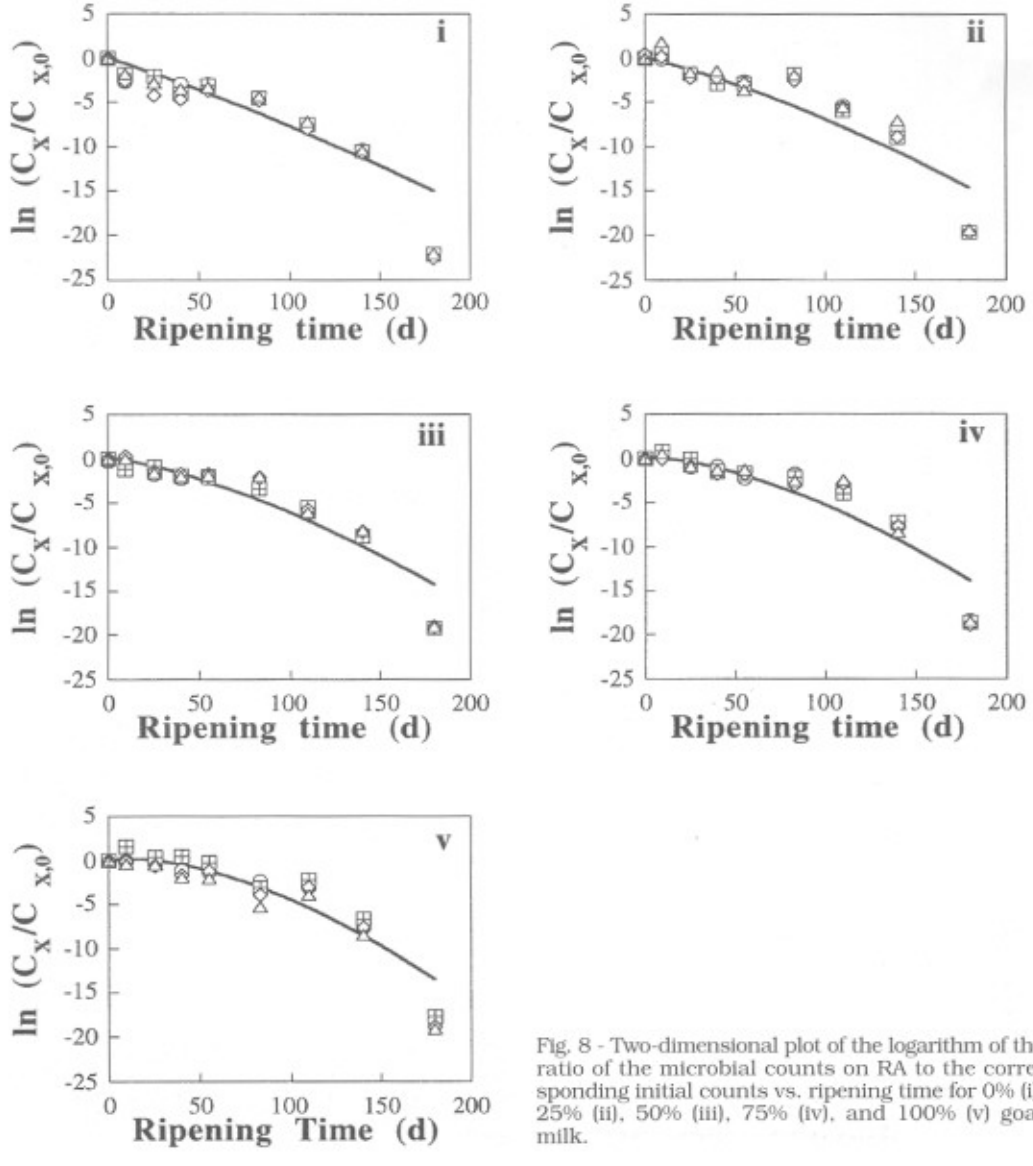

Fig. 8 - Two-dimensional plot of the logarithm of the ratio of the microbial counts on RA to the corresponding initial counts vs. ripening time for 0% (i), 25% (ii), 50% (iii), 75% (iv), and 100% (v) goat milk.

numbers of staphylococci increased slightly during the first days, stabilized from that time until 60 d of ripening, and then underwent a considerable decrease except for the 100C cheese, in which the counts of staphylococci tended to consistently decrease after 9 d of ripening (Fig. 2). For the 50C and 100C cheeses, staphylococci could not be detected after six months of ripening, although the same conclusion is unexpectedly not valid for the 75C cheese. Although BPM is a selective medium for *Staph. aureus*, the growth of coagulase-negative staphylococci was able to be observed. Scarce levels of *Staph. aureus*

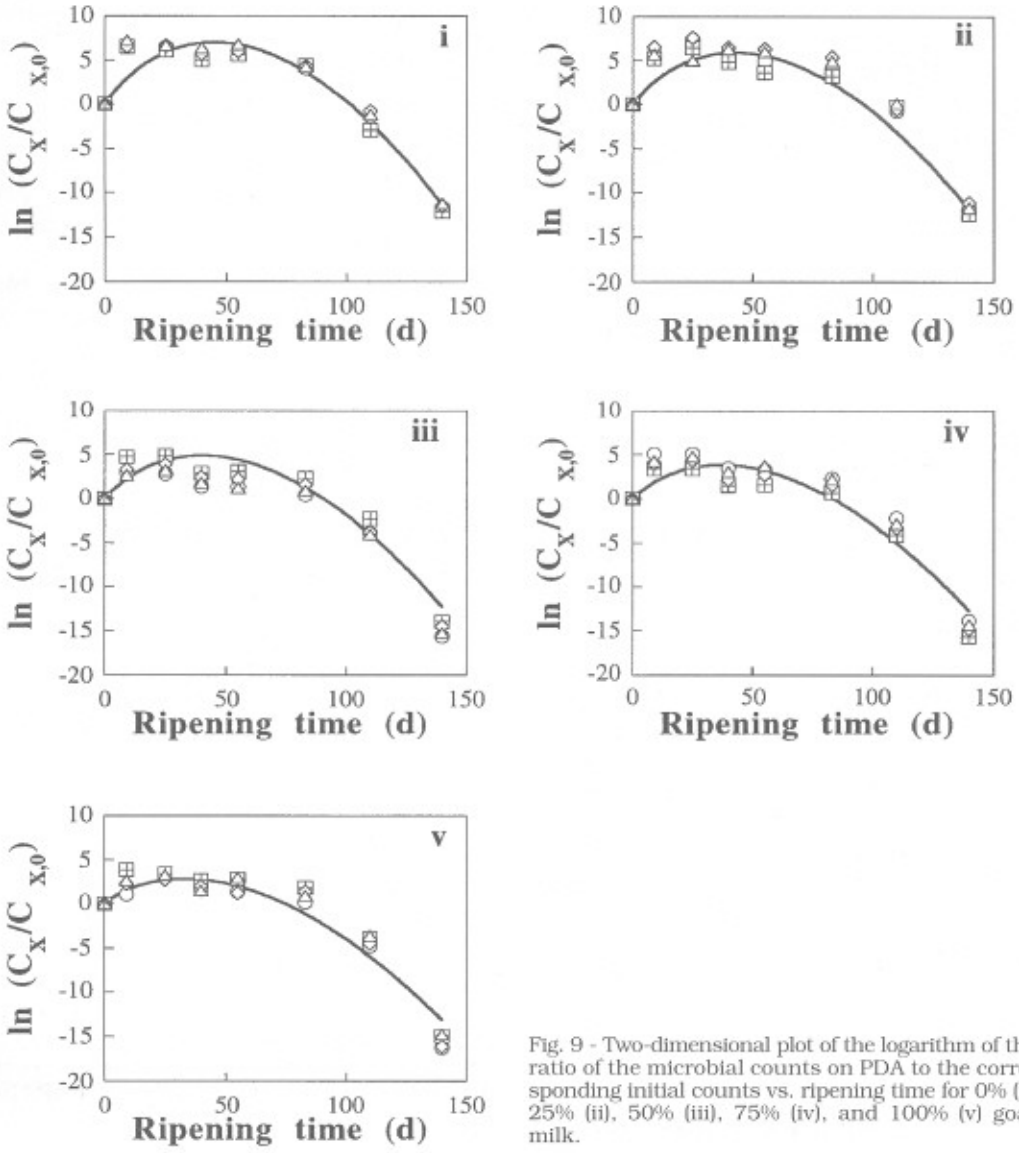

Fig. 9 - Two-dimensional plot of the logarithm of the ratio of the microbial counts on PDA to the corresponding initial counts vs. ripening time for 0% (i), 25% (ii), 50% (iii), 75% (iv), and 100% (v) goat milk.

were also reported for such artisanal cheeses as La Serena, a cheese manufactured with raw ewe milk (SANCHEZ-REY et al., 1993).

Initial counts of Enterobacteriaceae were in general high, thus providing an indication of the poor microbiological quality of the raw milk from the small ruminants and/or poor hygienic manufacturing conditions in the production of Picante cheese. The counts were different from cheese to cheese, ranging from 10^8 cfu/g for the 0C cheese to 10^6 cfu/g for the 100C cheese. These values decreased sharply until virtually disappearing at ca. 80 (0C, 25C, and 100C) or ca. 100 d (50C and 75C) as shown in Fig. 3. A similar decreasing tendency for Enterobacteriaceae counts was reported by GAYA et al. (1983). Different proportions of goat and ewe milk in cheese yield different counts in BPM and VRBGA, which is supported by the results in Table 3.

The major groups present throughout the ripening of Picante cheese were undoubtedly lactic acid bacteria and yeasts. Changes in M17A counts (viz. lactococci) are consistent with those reported for other artisanal cheeses, where again such microbes are one of the major contributors to the microflora prevailing during ripening (FONTECHA et al., 1990). During the ripening period, the M17A counts were above 10^8 cfu/g until approximately 140 d, and then suddenly decreased to 10^6 cfu/g during the last 40-d period (Fig. 4). The high values obtained for counts on M17A, mainly at 180 d of ripening, are somewhat surprising because, at this stage, Picante cheese has extremely high NaCl values (approximately 12%) and very low a_w values (below 0.80), both quite unfavorable conditions for lactococcus growth. One possible justification could be that M17A is not completely selective towards growth of lactococci, since other lactic acid bacteria may at times be observed (TORNADIJO et al., 1993); this hypothesis was confirmed *a posteriori* by analyses of isolates from M17A which confirmed the presence of enterococci. Results obtained by FONTECHA et al. (1990) working with artisanal cheeses made from goat and ewe milk have shown that the growth of enterococci followed approximately the same pattern as that of lactococci, i.e. high values early in the ripening period followed by a slow decrease. According to LLANO et al. (1992), enterococci remained at high values in the interior of Gamonedo cheese throughout ripening, suggesting that these bacteria could play an important role in the ripening of such cheese.

The lactobacilli group (mainly homofermentative) (RA) was initially present at high levels, ca. 10^8 cfu/g for all cheeses with the exception of the 0C cheese which had a count of 10^{10} cfu/g. A continuous decrease occurred during the first 3 months of ripening, after which a sharper decrease took place until virtually disappearing at 180 d (Fig. 5). Between 140 and 180 d of maturation, the NaCl content increased from values of 8-9% to 11.5-12% and the a_w dropped from values of 0.85-0.87 to 0.77-0.78 (Table 1). This could be related to the disappearance of lactobacillus strains, which are very labile to extreme environmental conditions. The ANOVA results (Table 3) also show that in the case of counts on RA, the milk composition and, to a greater extent, the ripening time factor have significant effects, whereas for the counts on M17A the effect of time seems to clearly prevail.

In all cheeses, large increases (in some cases above two orders of magnitude, from 10^6 to 10^8 cfu/g) in the levels of yeasts during the first days could be detected. These levels were maintained during the first month, and then dropped so that yeasts could not be detected after 140 d of ripening (Fig. 6). Growth of molds was seldom observed during the incubations on PDA. The yeasts found in

Picante cheese are essentially non-fermentative and the majority utilize lactic acid. Yeasts have been implicated in the second stage of maturation (ROHM et al., 1992) as well as in the production of flavour compounds resulting from their proteolytic and lipolytic activities (NUÑEZ et al., 1981). The ANOVA results pertaining to the presence of yeasts in the various cheeses throughout the ripening period (Table 3) show that the composition and, to a greater extent, the time factor had significant effects.

The pH in all cheeses dropped after salting. This decrease was due mainly to the metabolic activity of the lactic acid group of microorganisms. After that, there was a tendency of both inner and outer pH to increase throughout the ripening process (Table 1), attaining values of 5.77-5.89 and 6.14-6.32 at 180 d, respectively. The lower value of the inner pH is likely related to the environmental conditions prevailing in the interior of the cheese, where a more intensive lactic acid fermentation occurs than on the surface.

The NaCl content of Picante cheese is, on average, rather high; it attains values from 3%(w/w) after the first salting to 8%(w/w) after the second salting, and such high values are confirmed by the corresponding high values of ash content (Table 1). The increase in salt content that occurs between 140 and 180 d of ripening is probably related to the dehydration of the cheese (Fig. 1), which, due to the molecular nature of the salt migration requiring a liquid continuum, is likely to lead to supersaturation conditions. A concomitant decrease in the moisture content and water activity was observed throughout the ripening process, reaching very low values at 180 days of ripening (Table 1).

Table 4 indicates that in the evolution of both pH values and NaCl content, the factor time clearly has a much more significant effect than the composition (in the case of NaCl content, it was actually found that the composition is not statistically significant).

With respect to the semiempirical models considered, it is interesting to note that in general they provide good fit to the experimental data on the microbial counts for all families and are able to simulate local maxima wherever they exist; the fit is particularly good for the microbial strains grown on PDA (Fig. 9). These models are important because, to some extent, they allow the evolution of the microbial ecology to be predicted, a basic step for any attempt to standardize and eventually improve the manufacture of Picante cheese. It is remarkable that physico-chemical constraints are not violated by the estimates of the ε parameters, especially knowing that such estimates are obtained via a purely statistical route; viz. ε_2 is always positive, as expected from its definition cou-

Table 5 - Estimates of parameters in the model denoted as eq. (6).

Medium	ε_0 (d^{-1})	ε_1 (d^{-1})	ε_2 ($d^{-3/2}$)	ε_3 ($d^{-3/2}$)
PDA	-0.4639	0.002092	0.04610	-0.0001663
VRBGA	-0.1866	-0.0008444	0.0004662	0.00002598
BPM	-0.1547	0.001348	0.01381	0.00008519
RA	0.05947	-0.001037	0.001786	0.00007106
PCA	-0.08170	0.0007932	0.008495	-0.00004680
M17A	0.005451	-0.00001236	0.001104	0.000004845

pled with eqs. (3)-(5). In general, the trend for the number of viable microorganisms is to decrease with ripening time; after an initial transition period (which may even lead to increases in the numbers of viable microorganisms), such decreasing trend becomes essentially exponential as ripening time approaches completion. The dependence of the microecology of Picante on the composition of the cheesemaking milk (i.e. a gradual variation between the two limits corresponding to pure ewe and pure goat milk) was also somewhat expected.

Knowledge of the rates of reduction of viable counts of the various microbial families in Picante is crucial if educated attempts to establish microbiological safety for consumption are sought. The modelling procedure described above allows one to roughly anticipate when, within the ripening period, a Picante cheese will contain such potentially harmful microrganisms as staphylococci and Enterobacteriaceae at levels sufficiently below the safety threshold. Although claims may be raised that further work is warranted in order to determine whether such microbiological reductions also affect the perceived quality of the cheese, it should be noted that panel tasting of Picante cheese at intermediate stages of ripening would be difficult and possibly dangerous in view of the high levels of potential pathogens arising from the compulsory manufacture with raw milk. On the other hand, the composition of the milk mixture (in terms of volumetric proportions of ewe and goat milk) does not affect, on statistical grounds, the overall sensory characteristics of the final cheese and this realization suggests that attempts to improve the organoleptic profile of Picante cheese should not consider milk composition as a first priority.

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